03-30-00

A

Practitioner's Docket No. 49673 **PATENT**

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. § 601, 7th ed.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Paul PROOST, Sofie STRUYF and Jo VAN DAMME

WARNING: *37 C.F.R. § 1.41(a)(1) points out:*

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): AMINO-TERMINALLY TRUNCATED MCP-2 AS CHEMOKINE ANTAGONISTS

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date March 28, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number TB553892405US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Peter F. Corless
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

1. Type of Application

This new application is for a(n)

(check one applicable item below)

Original (nonprovisional)

☐ Design

☐ Plant

WARNING: ***Do not*** *use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: ***Do not*** *use this transmittal for the filing of a provisional application.*

NOTE: If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

☐ Divisional.

☒ Continuation.

☐ Continuation-in-part (C-i-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

NOTE: A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

NOTE: If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

WARNING: *When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application **must** be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).*

☒ The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

__14__ Pages of specification

__2__ Pages of claims

__4__ Sheets of drawing

WARNING: ***DO NOT*** *submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).*

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

☐ formal

☐ informal

B. Other Papers Enclosed

____ Pages of declaration and power of attorney

__1__ Pages of abstract

__3__ Other Pages of Sequence Listing

4. Additional papers enclosed

☐ Amendment to claims

☐ Cancel in this applications claims ____________ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☐ Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

☐ Preliminary Amendment

☐ Information Disclosure Statement (37 C.F.R. § 1.98)

☐ Form PTO–1449 (PTO/SB/08A and 08B)

☐ Citations

☐ Declaration of Biological Deposit

☐ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.

☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

☐ Special Comments

Other

5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)-(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).

☐ Enclosed

Executed by

(check **all** *applicable boxes)*

☐ inventor(s).

☐ legal representative of inventor(s). 37 C.F.R. §§ 1.42 or 1.43.

☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

☒ Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

☐ Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

☐ Showing that the filing is authorized.
(not required unless called into question. 37 C.F.R. § 1.41(d))

6. Inventorship Statement

WARNING: *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

☐ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

☐ is submitted.

☐ will be submitted.

7. Language

NOTE: An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of $130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

☒ English

☐ Non-English

☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

☒ An assignment of the invention to Applied Research Systems of Curacao Netherlands Antilles

☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

☒ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

WARNING: *A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.*

9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. No.	Filed
Europe	97116863.8	29 September 1997
Europe	97122471.2	19 December 1997
Europe	98104216.1	10 March 1998

from which priority is claimed

☐ is (are) attached.

☐ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. ☒ Regular application

CLAIMS AS FILED				
Number filed	Number Extra		Rate	Basic Fee 37 C.F.R. § 1.16(a) $760.00
Total Claims (37 C.F.R. § 1.16(c))	15 − 20 =	×	$ 18.00	
Independent Claims (37 C.F.R. § 1.16(b))	3 − 3 =	×	$ 78.00	
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))		+	$260.00	260.00

☐ Amendment cancelling extra claims is enclosed.

☐ Amendment deleting multiple-dependencies is enclosed.

☐ Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).

Filing Fee Calculation $ 950.00

B. ☐ Design application
($310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation $_____

C. ☐ Plant application
($480.00—37 C.F.R. § 1.16(g))

Filing fee calculation $_____

11. Small Entity Statement(s)

☐ Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can* ***unequivocally*** *make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).*

(complete the following, if applicable)

☐ Status as a small entity was claimed in prior application

_____ / _____, filed on _____, from which benefit is being claimed for this application under:

35 U.S.C. § ☐ 119(e),
☐ 120,
☐ 121,
☐ 365(c),

and which status as a small entity is still proper and desired.

☐ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A, B** or **C** above)

$_____

NOTE: Any excess of the full fee paid will be refunded if small entitiy status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

12. Request for International-Type Search (37 C.F.R. § 1.104(d))

(complete, if applicable)

☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

☒ Not Enclosed

☒ No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)

☐ Enclosed

☐ Filing fee $ _____

☐ Recording assignment
($40.00; 37 C.F.R. § 1.21(h))
(See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION".) $ _____

☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached
($130.00; 37 C.F.R. §§ 1.47 and 1.17(i)) $ _____

☐ For processing an application with a specification in a non-English language
($130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k)) $ _____

☐ Processing and retention fee
($130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l)) $ _____

☐ Fee for international-type search report
($40.00; 37 C.F.R. § 1.21(e)) $ _____

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total fees enclosed $_____

14. Method of Payment of Fees

☐ Check in the amount of $_____

☐ Charge Account No. _____ in the amount of $_____.

A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: *If no fees are to be paid on filing, the following items should not be completed.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

☐ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. _____:

☐ 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)

☐ 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☐ 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

☐ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).

☐ 37 C.F.R. § 1.17 (application processing fees)

NOTE: ". . .A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

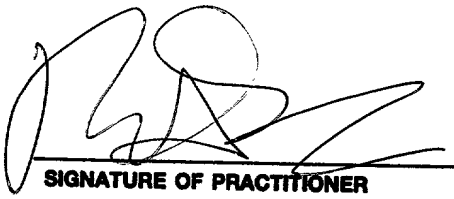
NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . . " From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

16. Instructions as to Overpayment

NOTE: ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☐ Credit Account No. _____

☐ Refund



SIGNATURE OF PRACTITIONER

Reg. No. 33,860

Peter F. Corless

(type or print name of attorney)

Tel. No. (617) 523-3400

Dike, Bronstein, Roberts & Cushman, LLP

130 Water Street

P.O. Address

Customer No.

Boston, MA 02109

☒ **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☐ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added _____

☐ Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added _____

☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added _____

☐ **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

☐ This transmittal ends with this page.

Practitioner's Docket No. 49673 **PATENT**

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 C.F.R. § 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

☒ Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. § 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☐ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
_____ /_____	_____ "
_____ /_____	_____ "
_____ /_____	_____ "

B. 35 U.S.C. §§ 120, 121 and 365(c)

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

☒ "This application is a

- ☒ continuation
- ☐ continuation-in-part
- ☐ divisional

of copending application(s)

☐ application number 0 /_____ filed on _____"

☒ International Application PCT/EP98/06142 filed on 28 September 1998 and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectivley. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

☐ "The nonprovisional application designated above, namely application _____ / _____, filed _____, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
_____ /_____	_____"
_____ /_____	_____"
_____ /_____	_____"

☐ Where more than one reference is made above, please combine all references into one sentence.

18. Relate Back—35 U.S.C. § 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

International	PCT/EP98/06142	September 28, 1998
Country	Appln. no.	Filed on

The certified copy(ies) has (have)

☐ been filed on _____, in prior application 0 /_____, which was filed on _____.

☐ is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may **not** be relied on without any need to file a certified copy of the priority application **in the continuing application.** This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

A. ☐ Extension of time in prior application

*(This item **must** be completed and the papers filed **in the prior application,** if the period set in the prior application has run.)*

☐ A petition, fee and response extends the term in the pending **prior** application until _____.

☐ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the pending **prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) ☐ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

- ☐ the same.
- ☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

_____

(type name(s) of inventor(s) to be deleted)

(b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

- ☐ the same.
- ☐ the following additional inventor(s) have been added:

_____

(type name(s) of inventor(s) to be added)

(c) The inventorship for all the claims in this application are

- ☐ the same.
- ☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made
  - ☐ is submitted.
  - ☐ will be submitted.

21. Abandonment of Prior Application *(if applicable)*

☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: *"The claims of a new application may be finally rejected in the first Office action in those situations where (A) the new application is a continuing application of, or a substitute for, an earlier application, and (B) all the claims of the new application (1) are drawn to the same invention claimed in the earlier application, and (2) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." M.P.E.P., § 706.07(b), 7th ed.*

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 C.F.R. § 1.28(a))

☐ Applicant has established small entity status by the filing of a statement in parent application /_____ on _____ .

☐ A copy of the statement previously filed is included.

WARNING: *See 37 C.F.R. § 1.28(a).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 7th ed. (emphasis added).*

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this
(check one of the following)

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

Docket No. 49673
Express Mail Label No. TB553892405US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
NEW PATENT APPLICATION

TITLE: AMINO-TERMINALLY TRUNCATED MCP-2 AS CHEMOKINE ANTAGONISTS

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AMINO-TERMINALLY TRUNCATED MCP-2 AS CHEMOKINE ANTAGONISTS

FIELD OF THE INVENTION

The present invention relates to amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, and pharmaceutical compositions comprising them.

BACKGROUND OF THE INVENTION

Chemokines constitute a family of small pro-inflammatory cytokines with leukocyte chemotactic and activating properties. Depending on the position of the first cysteines, the chemokine family can be divided in C-C, C-X-C and C-X_3-C chemokines (Baggiolini M. et al., 1994; Baggiolini M. et al., 1997 and Taub D. et al., 1996).

Many C-X-C chemokines such as interleukin-8 (IL-8) are chemotactic for neutrophils, while C-C chemokines, such as monocyte chemotactic protein-3 (MCP-3), are active on a variety of leukocytes including monocytes, lymphocytes, eosinophils, basophils, NK cells and dendritic cells.

The NH_2-terminal domain of chemokines is involved in receptor-binding and NH_2-terminal processing can either activate chemokines or render chemokines completely inactive.

The C-X-C chemokine platelet basic protein becomes a neutrophil chemotactic peptide (NAP-2) only after removal of the 24 NH_2-terminal residues (Walz A. et al., 1989 and Van Damme J. et al., 1990).

Deletion of up to 8 NH_2-terminal residues from IL-8 results in an enhanced chemotactic activity, but further cleavage of the Glu-Leu-Arg motif, which is located in front of the first Cys in all neutrophil chemotactic C-X-C chemokines, causes complete inactivation (Clark-Lewis I. et al., 1991).

Similar NH_2-terminal proteolysis (up to 8 amino acids) of another C-X-C chemokine, granulocyte chemotactic protein-2 (GCP-2), has no effect on the neutrophil chemotactic activity (Proost P.et al, 1993a).

The synthetical C-C chemokines MCP-1, MCP-3 and RANTES missing the 8 to 9 NH_2-terminal amino acids are inactive on monocytes and are useful as receptor antagonists (Gong J. et al., 1996; and Gong J. et al., 1995).

Extension of RANTES with one methionine results in complete inactivation of the molecule and Met-RANTES behaves as an antagonist for the authentic RANTES (Proudfoot A.E. et al., 1996).

The clone of human MCP-2 (Monocyte Chemoattractant Protein–2) has been isolated by differential library screening with cDNA probes derived from stimulated versus resting peripheral blood lymphocytes (PBL) (it was initially called "HC14", Chang H. C. et al., 1989). The cDNA-derived protein sequence was identical to that of purified natural MCP-2; however, a putative allelic variant has also been isolated, in which Gln 46 replaces Lys 46 (Van Coillie et al., 1997).

MCP-2 has also been synthesized by solid–phase chemistry (Proost P. et al., 1995).

DESCRIPTION OF THE INVENTION

The main object of the present invention are amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity

More particularly, one object of the present invention is MCP-2 (6-76), which is MCP-2 lacking the 1-5 NH_2-terminal amino acids, as shown in Figure 1 and in SEQ ID NO: 3 or SEQ ID NO: 4.

Such amino-terminally truncated MCP-2 of the invention can be in a glycosylated or non-glycosylated form.

The term "chemokine antagonist" means 'which acts as antagonist to the mature full-length naturally-occurring chemokines'.

Another object of the invention are the DNA molecules comprising the DNA sequences coding for the amino-terminally truncated MCP-2 of the invention, including nucleotide sequences substantially the same.

"Nucleotide sequences substantially the same" includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences.

The invention also includes expression vectors which comprise the above DNAs, host-cells transformed with such vectors and a process of preparation of such amino-terminally truncated MCP-2 of the invention, through the culture in appropriate culture media of said transformed cells.

The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable plasmid. Once formed, the expression vector is introduced into a suitable host cell, which then expresses the vector(s) to yield the desired protein.

Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g. yeasts, insect or mammalian cells) or prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should also comprise specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process.

There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived form viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the protein of the invention is inserted into vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell.

The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a auxotropic host, biocide resistance, e.g. antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells, that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation,

protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

The amino-terminally truncated MCP-2 of the invention may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, utilizing automated solid-phase peptide synthesizers followed by chromatographic purification.

The chemokines of the invention may, for example, be synthesized by Fmoc (9-fluorenylmethoxycarbonyl), tBoc (t-butoxycarbonyl) or any other comparable chemical synthesis with or without appropriate side-chain protection groups on the different amino acids. The amino acids with or without appropriate side-chain protection groups are preactivated - e.g. with HBTU/HOBt [2-(1H-Benzotriazole-1yl)-1,1,3,3-tetramethyl-uromium hexafluorophosphate/1-hydroxybenzotriazole) - and coupled to the growing peptide chain. Before the addition of the following residue, the protection group (e.g. Fmoc) is removed from the α-amino group. After synthesis, all protection groups are removed, the intact full length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding chemokines of the invention.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving

extraction, precipitation, chromatography, electrophoresis, or the like (see for example Proost P. et al., 1996). A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, or affinity for heparin, which bind the target protein and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by heparin or by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

The amino-terminally truncated MCP-2 of the invention are useful in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required. Examples of such diseases include: inflammatory diseases, angiogenesis- and hematopoiesis-related diseases, tumors, infectious diseases, including HIV, auto-immune diseases, atherosclerosis, pulmonary diseases and skin disorders.

Therefore, in a further aspect, the present invention provides the use of the protein of the invention in the manufacture of a medicament for the treatment of the above-mentioned diseases.

The medicament is preferably presented in the form of a pharmaceutical composition comprising the proteins of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions form yet a further aspect of the present invention.

A further embodiment of the invention is the method of treatment of the above-mentioned diseases comprising administering a pharmacologically active amount of the amino-terminally truncated MCP-2 of the invention to subjects at risk of developing such diseases or to subjects already showing such pathologies.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

DESCRIPTION OF THE FIGURES

Figure 1: it shows the amino acid sequence of MCP-2 and of its known variant. Signal sequences are reported in *italics*, whereas C -residues are in **bold**. Arrows indicate the

first amino acids of the amino-terminally truncated MCP-2(6-76) of the invention. Underlined is the amino acid, which is different in the MCP-2 variant.

Figure 2: SDS-PAGE of amino-terminally truncated MCP-2(6-76):

lane 1: natural MCP-2 (1-76, 100 ng/lane);

lane 2: natural MCP-2 (1-76, 30 ng/lane);

lane 3: natural MCP-2 (6-76, 30 ng/lane); and

lane 4: synthetic MCP-2 (1-76, 60 ng/lane).

Gels were run under reducing conditions and proteins were stained with silver.

Figure 3: it shows a comparison of the chemotactic potency of modified MCP-2 forms. Intact natural (nat) and synthetic (syn) MCP-2(1-76), NH_2-terminally truncated natural MCP-2(6-76) and COOH-terminally truncated synthetic MCP-2(1-74) were tested for chemotactic activity on THP-1 cells. Results represent the mean CI ± SEM from four or more independent experiments.

Figure 4: Natural MCP-2 is a weaker agonist than MCP-1 to mobilize calcium in monocytes. Intact MCP-2 (15, 50 and 150 ng/ml) dose-dependently increases the $[Ca^{2+}]_i$ in THP-1 cells. The result of one representative experiment out of two is shown.

EXAMPLES

EXAMPLE 1: Amino-terminally truncated MCP-2

Materials and methods

Chemokine and immunoassay

MCP-2 was synthesized and purified as described earlier (Proost P. et al.,1995).

Specific anti-human MCP-2 Ab were obtained from mice and affinity purified on a Sepharose column to which synthetic MCP-2 was coupled using the conditions provided by the manufacturer (CNBr activated Sepharose 4B, Pharmacia, Uppsala, Sweden).

ELISA plates were coated with the affinity purified anti-human MCP-2 and biotinylated anti-MCP-2 was used as the capturing Ab. The detection was performed with peroxidase labeled streptavidine and TMB. The detection limit for the MCP-2 ELISA was about 0.1 ng/ml.

Production and purification of MCP-2

Monocyte chemotactic proteins were purified from peripheral blood mononuclear cell-derived conditioned medium from 132 blood donations obtained from Blood Transfusion Centers of Antwerp and Leuven (Proost P. et al., 1996).

Erythrocytes and granulocytes were removed by sedimentation in hydroxyethyl starch (Fresenius AG, Bad Homburg, Germany) and by gradient centrifugation in a sodium metrizoate solution (Lymphoprep; Nyegaard, Oslo Norway).

Mononuclear cells ($60x10^9$ cells) were incubated ($5x10^6$ cells/ml) with 10 µg/ml Con A and 2 µg/ml of LPS. After 48 to 120 h, conditioned medium was collected and kept at -20 °C until purification.

Natural MCP-2 was purified in a four step purification procedure as previously described (Proost P. et al., 1996).

Briefly, the conditioned medium was concentrated on controlled pore glass or silicic acid and partially purified by affinity chromatography on a heparin-Sepharose column (Pharmacia).

Fractions containing MCP-2 immunoreactivity were further purified by Mono S (Pharmacia) cation exchange chromatography and eluted in a NaCl gradient at pH 4.0.

Natural MCP-2 were purified to homogeneity through RP-HPLC on a C-8 Aquapore RP-300 column (Perkin Elmer, Norwalk CT) equilibrated with 0.1 % trifluoroacetic acid TFA). Proteins were eluted in an acetonitrile gradient.

Biochemical characterization of MCP-forms by SDS-PAGE, amino acid sequence analysis and mass spectrometry

The purity of column fractions was examined by SDS-PAGE under reducing conditions on Tris/tricine gels (Proost P. et al., 1996). Proteins were stained with silver and the following relative molecular (*Mr*) markers were used: OVA (*Mr* 45,000), carbonic anhydrase (*Mr* 31,000), soybean trypsin inhibitor (*Mr* 21,500), β-lactoglobulin (*Mr* 18,400), lysozyme (*Mr* 14,400) and aprotinin (*Mr* 6,500).

The NH_2-terminal sequence of purified chemokines was determined by Edman degradation on a pulsed liquid 477A/120A protein sequencer (Perkin Elmer) with N-methylpiperidine as a coupling base. Blocked proteins were cleaved between Asp and

Pro in 75 % formic acid for 50 h. The formic acid digest was sequenced without further purification.

The *Mr* of MCP-2 was determined by matrix-assisted laser desorption ionization/time of flight-mass spectrometry (MALDI/TOF-MS) (Micromass TofSpec, Manchester, UK). Alpha-cyano-4-hydroxycinnamic acid and cytochrome C were used as matrix and internal standard, respectively.

Detection of chemotactic activity

MCP-2 was tested for its chemotactic potency on freshly purified monocytes ($2x10^6$ cells/ml) or monocytic THP-1 cells ($0.5x10^6$ cells/ml; 2 days after subcultivation) in the Boyden microchamber using polyvinylpyrrolidone-treated polycarbonate membranes with 5 μm pore size.

Samples and cells were diluted in HBSS (Life technologies/Gibco BRL, Paisley, Scotland) supplemented with 1 mg/ml human serum albumin (Red Cross Belgium). After 2 h incubation at 37 °C, the cells were fixed and stained with Diff-Quick staining solutions (Harleco, Gibbstown. NJ) and the cells that migrated through the membranes were counted microscopically in ten oil immersion fields at 500-fold magnification.

The chemotactic index (CI) of a sample (triplicates in each chamber) was calculated as the number of cells that migrated to the sample over the number of cells that migrated to control medium (Van Damme J. et al., 1992).

For desensitization experiments, cells were incubated with biologically inactive chemokine-variants for 10 min. at 37°C, before they were added to the upper well of the Boyden microchamber. The % inhibition of the CI was calculated using the CI from HBSS treated cells towards the sample as a reference value.

Detection of intracellular Ca^{2+} concentrations

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured as previously described (Wuyts A. et al., 1997). Purified monocytes or THP-1 cells (10^7 cells/ml) were incubated in Eagle's Minimum Essential Medium (EMEM, Gibco) + 0.5 % FCS with the fluorescent indicator fura-2 (fura-2/AM 2.5 μM; Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01 % Pluronic F-127 (Sigma, St Louis MO).

After 30 min at 37 °C the cells were washed twice and resuspended at 10^6 cells/ml in HBSS with 1 mM Ca^{2+} and 0.1 % FCS (buffered with 10 mM Hepes/NaOH at pH 7.4). The cells were equilibrated at 37 °C for 10 min before fura-2 fluorescence was measured in a LS50B luminescence spectrophotometer (Perkin Elmer).

Upon excitation at 340 and 380 nm, fluorescence was detected at 510 nm. The $[Ca^{2+}]_i$ was calculated from the Grynkiewicz equation (Grynkiewicz et al, 1985). In order to determine R_{max} the cells were lysed with 50 µM digitonin. Subsequently, the pH was adjusted to 8.5 with 20 mM Tris and R_{min} was obtained by addition of 10 mM EGTA to the lysed cells. The K_d used was 224 nM.

For desensitization experiments, monocytes or THP-1 cells were first stimulated with buffer, chemokine or chemokine antagonist at different concentrations. As a second stimulus, MCP-2 was used at a concentration inducing a significant increase in the $[Ca^{2+}]_i$ after prestimulation with buffer. The second stimulus was applied 2 min after addition of the first stimulus. The percentage inhibition of the $[Ca^{2+}]_i$ increase in response to the second stimulus was calculated comparing the signal after prestimulation with chemokine or chemokine antagonist with the signal after addition of buffer.

Results

Isolation of post-translationally modified MCP-2 forms

A specific and sensitive ELISA was used to trace different MCP-2 forms produced by peripheral blood mononuclear cells stimulated with mitogen and endotoxin.

The conditioned medium was purified according to a standard isolation procedure (Proost P. et al., 1996), including adsorption to controlled pore glass and heparin Sepharose chromatography.

Subsequently, purification by FPLC mono S cation exchange chromatography was carried out and then a further purification step with C-8 RP HPLC was applied. Molecular masses were measured by SDS-PAGE and by MALDI/TOF-MS.

Different forms of MCP-2 were isolated: in addition to the authentic 7.5 kDa MCP-2(1-76), an NH_2-terminally truncated 7 kDa form of MCP-2 missing five residues [MCP-2(6-76)] was purified to homogeneity by RP-HPLC and identified by amino acid sequence analysis (Fig. 2). MALDI/TOF-MS (Table I) yielded a molecular mass of 8881

Da for intact MCP-2 (theoretical *Mr* of 8893 Da), whereas for the MCP-2(6-76) a molecular mass of 8365 Da was measured, confirming the deletion of the five NH_2-terminal amino acids (theoretical *Mr* of 8384 Da). Functional comparison of these natural MCP-2 forms in the THP-1 chemotaxis assay showed that intact MCP 2 is still active at 5 ng/ml, whereas truncated MCP-2(6-76) remains devoid of chemotactic activity when tested at a concentration range from 0.6 to 60 ng/ml (Fig. 3). Intact natural MCP-2 was also compared in potency with the synthetical MCP-2(1-76) and a COOH-terminally truncated synthetical form (Proost P. et al., 1995) missing two residues [MCP-2(1-74)].

The minimal effective chemotactic concentration of these forms was also found to be 5 ng/ml (Fig. 3). Although in chemotaxis assays the specific activity of natural intact MCP-1 and MCP-2 is comparable (Van Damme J, et al., 1992), the calcium mobilizing capacity of MCP-2 is still a matter of debate.

However, in Ca^{2+} -mobilization experiments, the minimal effective dose for both natural or synthetic MCP-2(1-76) was 10-fold higher compared to that of natural intact MCP-1(1-76) (Fig. 4), whereas MCP-2(6-76) remained inactive.

Nevertheless, intact MCP-2 (50 ng/ml) was capable to desensitize for MCP-2 (15 ng/ml) and MCP-3 (10 ng/ml) yielding 52% and 45% inhibition of chemotaxis, respectively.

Due to this lower specific activity of MCP-2 in Ca^{2+} assays, desensitization of chemotaxis by MCP-2(6-76) was performed in the Boyden microchamber. Since intact MCP-2 is reported to cross-desensitize with active MCP-1, MCP-2 and MCP 3 in the monocyte chemotaxis assay (Sozzani S. et al., 1994), we investigated whether natural, inactive MCP-2(6-76) could also desensitize for MCP-1, MCP-2, MCP-3 and RANTES (Table II). Pre-incubation of THP-1 cells with 100 ng/ml of inactive MCP-2(6-76) could already significantly inhibit chemotaxis induced by 10 ng/ml of MCP-1 (63 %), 5 ng/ml of MCP-2 (75%), 30 ng/ml of MCP-3 (62 %) and 100 ng/ml of RANTES (75%). Moreover, chemotaxis in response to 3 times lower concentrations of the respective MCPs was completely (91-100 %) inhibited by 100 ng/ml MCP-2(6-76). Furthermore, at a concentration as low as 10 ng/ml, MCP-2(6-76) was still able to significantly inhibit the chemotactic activity induced by MCP-1 (3 ng/ml), MCP-2 (1.5 ng/ml) or

MCP 3 (10 ng/ml) and RANTES (30 ng/ml). Taken together, MCP-2(6-76) is produced naturally, is inactive as a chemoattractant and antagonizes several C-C chemokines, the effect being most predominant for MCP-3.

TABLE I

Biochemical characterization of natural forms of MCP-2. NH_2-terminal amino acid sequence analysis and comparison of the experimental (SDS-PAGE and MALDI/TOF-MS) and theoretical Mr of C-8 RP-HPLC purified natural MCP-isoforms.

MCP-form	NH_2-terminal sequence	*Mr* (Da)		
		theoretical unglycosylated	SDS-PAGE	MALDI/TOF-MS
MCP-2 (1-76)	blocked	8893	7500	8881
MCP-2 (2-76)	SIPITCC	8384	7000	8365

TABLE II

MCP-2(6-76) desensitizes the monocyte chemotactic responses of MCP-1, MCP-2 MCP-3 and RANTES in the microchamber.

Chemokine [a]	Concentration	Antagonization of chemotactic response [b,c]		% Inhibition of chemotaxis
		buffer	100 ng/ml MCP-2(6-76)	
MCP-1	10	22.3 ± 7.9	8.3 ± 3.8	63 ± 21
	3	15.0 ± 8.0	1.3 ± 0.3	99 ± 1.0
MCP-2	5	36.0 ± 12.6	10.8 ± 6.1	75 ± 8.0
	1.5	6.7 ± 1.4	1.5 ± 0.3	91 ± 7.0
MCP-3	30	13.2 ± 0.4	6.0 ± 4.0	62 ± 31
	10	3.0 ± 1.5	<1	100 ± 0.0
RANTES	100	6.3 ± 0.8	2.6 ± 1.3	75 ± 19
	30	4.0 ± 0.8	1.5 ± 0.3	77 ± 16
		buffer	10 ng/ml MCP-2 (6-76)	
MCP-1	10	12.7 ± 2.3	10.5 ± 3.8	24 ± 1.8
	3	7.5 ± 0.0	3.0 ± 0.3	69 ± 4.0
MCP-2	5	38.0 ± 5.3	27.2 ± 4.9	30 ± 6.0
	1.5	18.3 ± 4.6	9.2 ± 1.4	45 ± 23
MCP-3	30	13.2 ± 1.9	8.0 ± 1.0	37 ± 19
	10	7.7 ± 1.4	1.7 ± 0.3	90 ± 6.0
RANTES	100	5.5 ± 0.6	5.8 ± 0.9	17 ± 7.0
	30	3.2 ± 0.7	2.5 ± 0.5	39 ± 18

[a] MCP-1, MCP-2, MCP-3 or RANTES were added as chemoattractants to the lower wells.

[b] the upper wells of the microchamber were filled with THP-1 cells preincubated with MCP-2(6-76) or with buffer

[c] mean CI ± SEM of 3 independent experiments

REFERENCES

Baggiolini M. et al., *Ann. Rev. Immunol.*, **55**, 97-179, 1994.

Baggiolini M. et al., *Ann. Rev. Immunol.*, **15**, 675-705, 1997.

Chang H. C. et al., *International Immunology*, **1**(4), 388-397, 1989.

Clark-Lewis I. et al., *J. Biol. Chem.*, **266**, 23128-23134, 1991.

De Meester I. et al., *J. Immunol. Methods* **189**, 99-10526, 1996.

Deng H. et al., *Nature.*, **381**, 661-666, 1996.

Gong J. et al. *J. Exp. Med.*, **181**, 631-640, 1995.

Gong J. et al., *J. Biol. Chem.* **271**, 10521-10527, 1996.

Grynkiewicz G. et al., *J. Biol. Chem.*, **260**, 3440, 1985.

Proost P.et al., *Biochemistry*, **32**, 10170-10177, 1993a.

Proost P. et al., *J. Immunol.*, **150**, 1000-1010, 1993.

Proost P. et al., *Cytokine*, **7**, 97-104, 1995.

Proost P. et al., *Methods: A companion to Methods in Enzymol.*, **10**, 82, 1996.

Proudfoot A. E. et al., *J. Biol. Chem.*, **271**, 2599-2603, 1996.

Sambrook et al, Molecular Cloning: A laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

Schols D. et al., *J. Exp. Med.*, **186**, 1383-1388, 1997.

Sozzani S. et al., *J. Immunol.*, **152**, 3615, 1994.

Taub D. et al., *Cytokine & Growth Factor Reviews*, **7**, 335-76, 1996.

Van Coillie E. et al., *Biochem. Biophys. Res. Commun.*, **231**, 726-730, 1997.

Van Damme J. et al., Eur. J. Biochem., **181**, 337-344, 1989.

Van Damme J. et al., *Eur. J. Immunol.*, **20**, 2113-8, 1990.

Van Damme J. et al., *J. Exp. Med.*, **176**, 59, 1992.

Walz A. et al., *Biochem. Biophys. Res. Commun.*, **159**, 969-75, 1989.

Wuyts A., et al., *Biochemistry* **36**, 2716-2723, 1997.

CLAIMS

1. Amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity.

2. Amino-terminally truncated MCP-2 according to claim 1, lacking NH_2-terminal amino acids corresponding to amino acid residues 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity.

3. Amino-terminally truncated MCP-2 according to claim 1, having the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

4. Amino-terminally truncated MCP-2 according to one or more of the preceding claims, in a glycosylated form.

5. DNA molecules comprising the DNA sequences coding for the amino-terminally truncated MCP-2 of the invention according to one or more of the preceding claims, including nucleotide sequences substantially the same.

6. An expression vector which comprises the DNA molecule of any claim 5.

7. A host cell comprising the expression vector of claim 5.

8. A recombinant process for preparing any of the proteins from claim 1 to 4, comprising culturing in an appropriate culture medium the cells of claim 6.

9. A protein according to any of the claims from 1 to 4 for use as medicament.

10. Use of a protein according to any of the claims from 1 to 4, in the manufacture of a medicament for the therapy and/or diagnosis of diseases, in which an antagonistic activity of the chemokine effects is required.

11. Use according to claim 10, in the manufacture of a medicament for the treatment of inflammatory diseases, HIV-infection, angiogenisis- and hematopoiesis-related diseases, and tumors.

12. A pharmaceutical composition comprising the protein according to any of the claims from 1 to 4 together with one or more pharmaceutically acceptable carriers and/or excipients.

ABSTRACT

The present invention relates to amino-terminally truncated MCP-2, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3, 1-4 or 1-5 of the naturally-occurring MCP-2 and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, and pharmaceutical compositions comprising them.

MCP-2

```
-23                          1    ↓
MKVSAALLCL LLMAATFSPQ GLAQPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP
KEAVIFKTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP
                                          76
```

MCP-2 Variant

```
-23                          1    ↓
MKVSAALLCL LLMAATFSPQ GLAQPDSVSI PITCCFNVIN RKIPIQRLES YTRITNIQCP
KEAVIFQTKR GKEVCADPKE RWVRDSMKHL DQIFQNLKP
                                          76
```

Figure 1

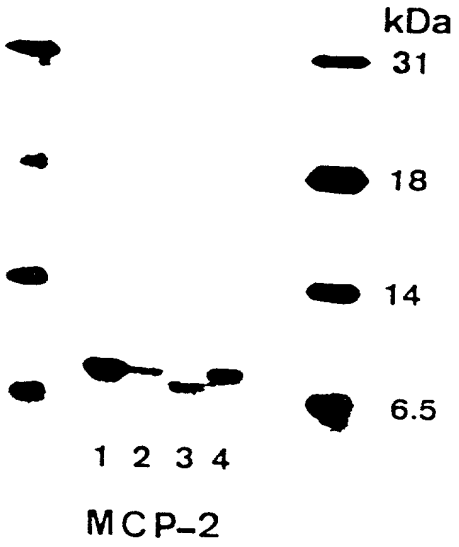

Figure 2

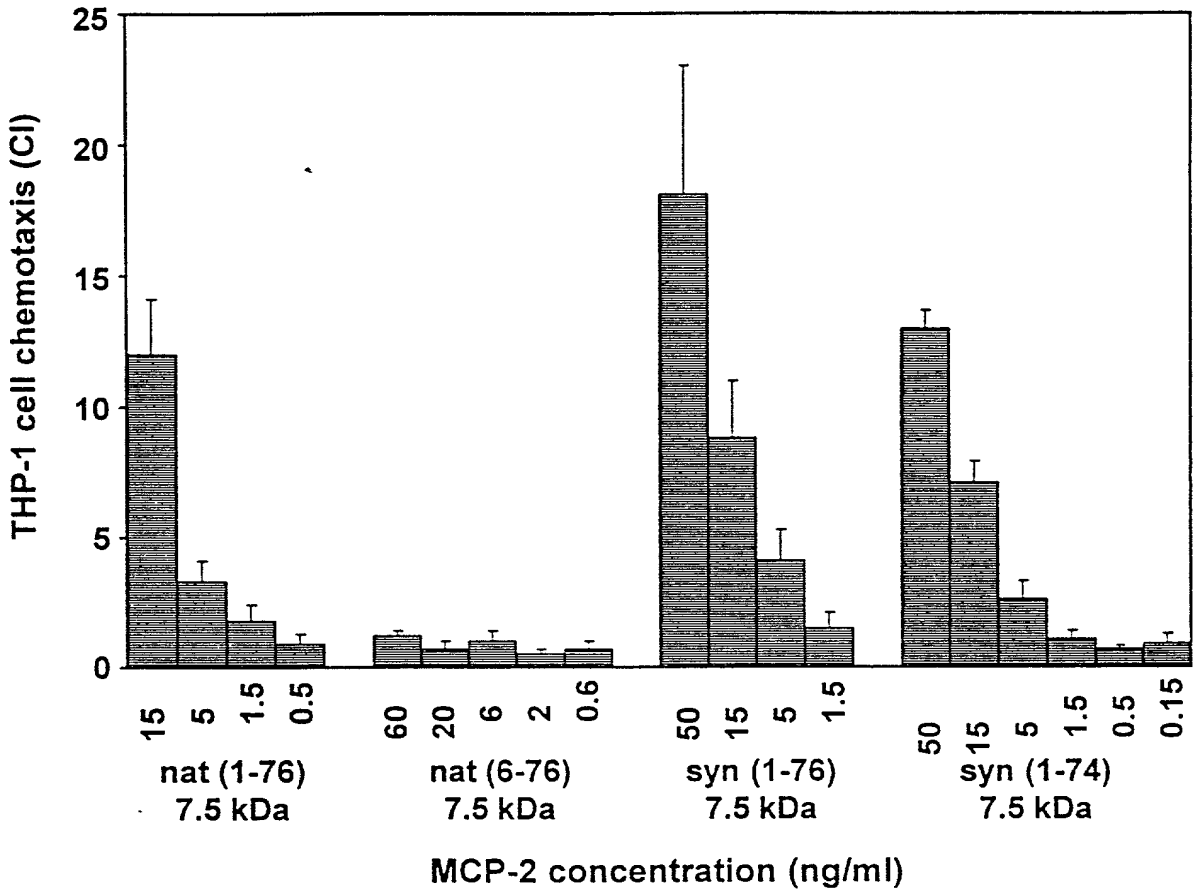
THP-1 cell chemotaxis (CI)
25
20
15
10
5
0
15
5
1.5
0.5
60
20
6
2
0.6
50
15
5
1.5
50
15
5
1.5
0.5
0.15
nat (1-76) 7.5 kDa
nat (6-76) 7.5 kDa
syn (1-76) 7.5 kDa
syn (1-74) 7.5 kDa
MCP-2 concentration (ng/ml)

Figure 3

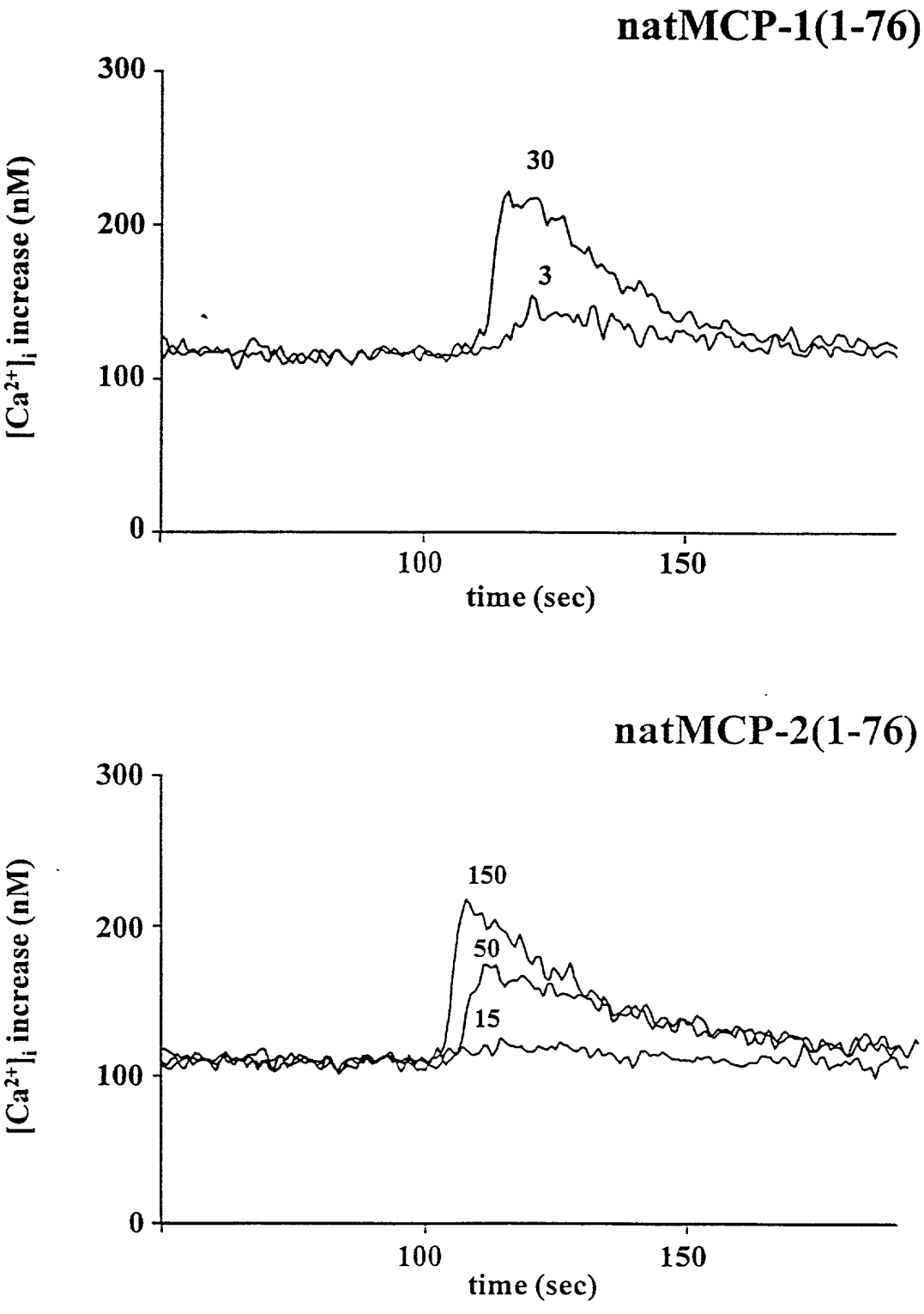
natMCP-1(1-76)
300
200
100
0
30
3
100
150
time (sec)
[Ca2+]i increase (nM)
natMCP-2(1-76)
300
200
100
0
150
50
15
100
150
time (sec)
[Ca2+]i increase (nM)

Figure 4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.
(B) STREET: 14 JOHN B. GORSIRAWEG
(C) CITY: CURACAO
(E) COUNTRY: THE NETHERLANDS ANTILLES
(F) POSTAL CODE (ZIP): NONE
(G) TELEPHONE: 639300
(H) TELEFAX: 614129

(ii) TITLE OF INVENTION: AMINO-TERMINALLY TRUNCATED MCP-2 AS CHEMOKINE ANTAGONISTS

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION:1..76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Met Ala Ala Thr
            -20                 -15                 -10

Phe Ser Pro Gln Gly Leu Ala Gln Pro Asp Ser Val Ser Ile Pro Ile
        -5                  1               5

Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu
10                  15                  20                  25

Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro Lys Glu Ala Val

                30                  35                  40
```

Ile Phe Lys Thr Lys Arg Gly Lys Glu Val Cys Ala Asp Pro Lys Glu
45 50 55

Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln Ile Phe Gln Asn
60 65 70

Leu Lys Pro
75

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION:1..76

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Met Ala Ala Thr
-20 -15 -10

Phe Ser Pro Gln Gly Leu Ala Gln Pro Asp Ser Val Ser Ile Pro Ile
-5 1 5

Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro Ile Gln Arg Leu
10 15 20 25

Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro Lys Glu Ala Val
30 35 40

Ile Phe Lys Thr Gln Arg Gly Lys Glu Val Cys Ala Asp Pro Lys Glu
45 50 55

Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln Ile Phe Gln Asn
60 65 70

Leu Lys Pro
75

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro
1               5                  10                  15

Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro
            20                  25                  30

Lys Glu Ala Val Ile Phe Lys Thr Lys Arg Gly Lys Glu Val Cys Ala
        35                  40                  45

Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln
    50                  55                  60

Ile Phe Gln Asn Leu Lys Pro
65                  70
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Ser Ile Pro Ile Thr Cys Cys Phe Asn Val Ile Asn Arg Lys Ile Pro
1               5                  10                  15

Ile Gln Arg Leu Glu Ser Tyr Thr Arg Ile Thr Asn Ile Gln Cys Pro
            20                  25                  30

Lys Glu Ala Val Ile Phe Lys Thr Gln Arg Gly Lys Glu Val Cys Ala
        35                  40                  45

Asp Pro Lys Glu Arg Trp Val Arg Asp Ser Met Lys His Leu Asp Gln
    50                  55                  60

Ile Phe Gln Asn Leu Lys Pro
65                  70
```